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Rescuing Biological Activity from Synthetic Phakellistatin 19

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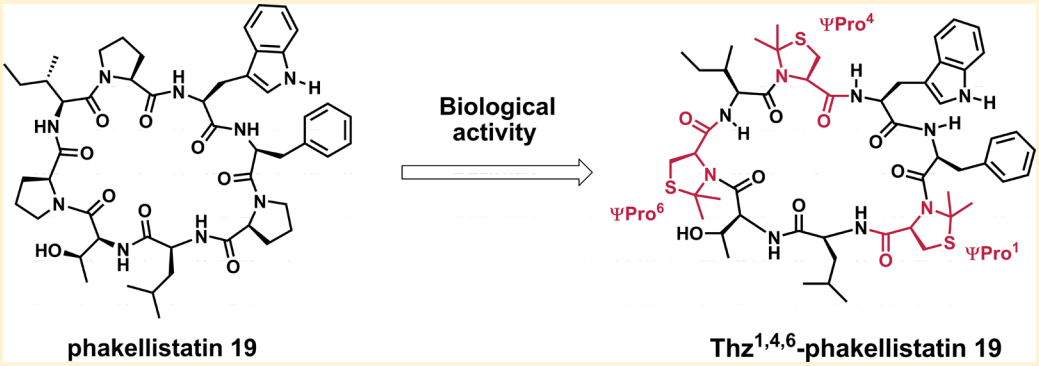
Supporting Information



ABSTRACT: Phakellistatins is one of the families of Pro-rich cyclic peptides whose synthetic counterparts have revealed cytotoxicities that differ greatly from those displayed by their corresponding natural ones. This is also the case of the last member isolated from this family, phakellistatin19, an octacyclopeptide containing three Pro moieties and a high percentage of apolar residues. Exhaustive NMR studies on the synthetic and natural phakellistatin 19 have been performed in order to find a plausible explanation for this intriguing behavior. Moreover, taking advantage of phakellistatin's framework, analogues with different *cis*/*trans* geometry at the key prolyl peptide bonds were designed, covering a promising conformational space that could not be reached by the natural peptide. By introduction of proline surrogates ($\Psi^{Me,Me}$pro residues) in phakellistatin 19, which effectively increases the percentage of *cis* conformation in the final peptides, this translates into enhanced biological activity, therefore "rescuing" an otherwise inactive cyclopeptide.

INTRODUCTION

Several families of proline-rich peptides isolated from marine sponges and displaying significant cytotoxicity, such as hymenamides,[1] stylopeptides,[2] axinellins,[3] axinastatins,[4] and phakellistatins,[5] have been isolated. All of these peptides share a number of structural features. In general, they comprise homodetic hepta- or octapeptides with an unusual percentage of Pro moieties, with high contents of apolar amino acids, including one or two aromatic residues, and with a significant structural analogy.

Nineteen cyclopeptides have been isolated so far from marine sponges of the genus *Phakellia*. They all consist of seven to 10 amino acids, including at least one Pro moiety, most of them having more than one. Of all the peptides described, four comprise the distinctive Pro-Pro track,[6] which represents a considerable synthetic challenge.

Phakellistatin 19 (Figure 1) is an octacyclopeptide containing three Pro moieties and a high percentage of apolar residues, including a Leu, an Ile, and a Phe residue. Its amino acid sequence greatly resembles that of phakellistatin 10[7] but with one single modification; the Val moiety is replaced by a Phe residue. Biological evaluation of natural phakellistatin 19 showed promising cytotoxic and antimitotic activity (see Table 2).

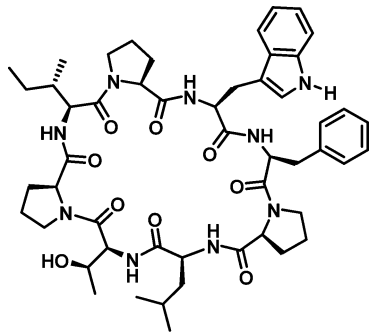


Figure 1. Chemical structure of phakellistatin 19.

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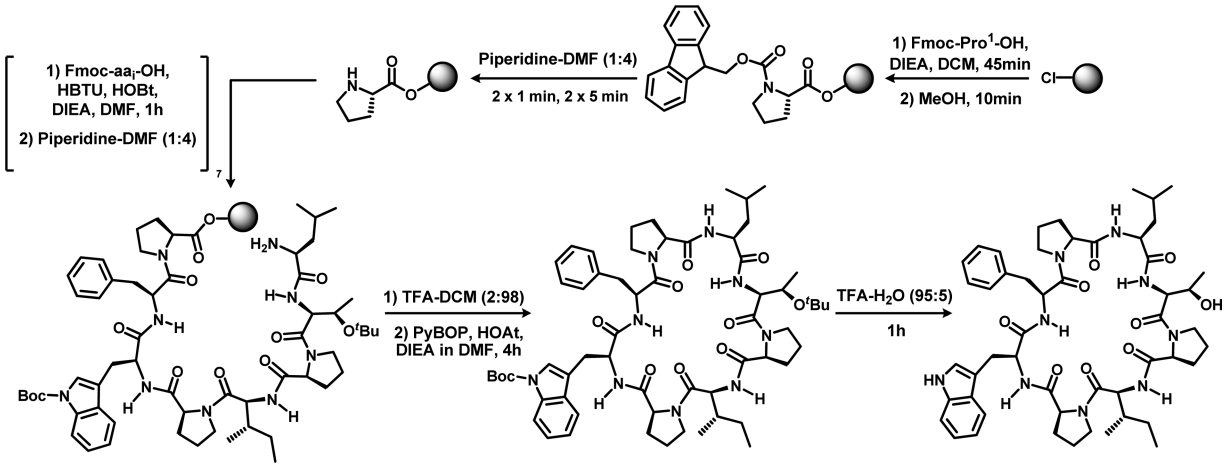
Scheme 1. Synthetic Scheme for Phakellistatin 19



Surprising behavior has been associated with phakellistatins and other proline-rich cyclopeptides such as axinastins[8] and stylopeptides.[2] Thus, after chemical and spectral validation by means of nuclear magnetic resonance (NMR), high performance liquid chromatography (HPLC), high-resolution mass spectrometry (HRMS), and Marfey's techniques, biological evaluation of synthetic peptides has revealed cytotoxicities that differ greatly from those displayed by their natural counterparts. This phenomenon has been widely reported by several groups working in this field and represents a scientific puzzle.[2,8,9] This is also the case of our synthetic phakellistatin 19.

RESULTS AND DISCUSSION

To date, two main hypotheses have been proposed to explain this biological incongruity. On the one hand, a number of authors argue that the presence of trace amounts of a highly cytotoxic contaminant that binds noncovalently to natural phakellistatins (or peptides from other families) would account for the biological activity of these compounds.[9] This cytotoxic agent would be present in a low percentage and would thus prevent its detection by NMR spectroscopy. In this sense, Pettit et al.[9c] proved that, indeed, nondetectable NMR contamination can cause biological activity. On the other hand, a conformational issue caused by the presence of a high percentage of Pro residues in quite a small cyclopeptide may also explain this intriguing biological behavior.

Finally, in our case, two other possible causes were discarded. Thus, the hypothesis of a stereochemical misassignment was abandoned after the synthesis and biological evaluation of 10 possible epimers of phakellistatin 19 (see Supporting Information).[10] Neither did preliminary chelation experiments account for the differences in cytotoxicity, as no effective chelation was detected by matrix-assisted laser desorption/ionization (MALDI) analysis (see Supporting Information).

Thus, our efforts were focused on performing a thorough NMR study of both the synthetic and natural samples in the search of conformational differences, without disregarding the possibility of an impurity present in the natural sample, which would be difficult to prove.

Synthesis of Phakellistatin 19. The linear precursor of phakellistatin 19 was synthesized on solid phase following the 9-fluorenylmethoxycarbonyl/*tert*-butyl (Fmoc/^{t}Bu) protection scheme and using the 2-chlorotrityl[11] chloride resin (2-CTC) as the polymeric support to minimize diketopiperazine (DKP) formation and to perform the "head-to-tail" cyclization in solution.

As the macrolactamization step poses the biggest synthetic challenge of an all-L-cyclopeptide synthesis, two cyclization/starting points were evaluated, namely, Leu(C)-(N)Thr and Pro(C)-(N)Leu. The first amide bond involved a β-branched residue, while the second linkage had a Pro at the *C*-terminus, which minimized racemization during cyclization but increased the risk of DKP formation during the assembly of the linear peptide. The two approaches were carefully examined. Use of the Pro-Leu linkage as the cyclization point rendered the desired product with overall better yields (Scheme 1).

1-[Bis(dimethylamino)methylene]-1*H*-benzotriazolium hexafluorophosphate 3-oxide (HBTU)/1-hydroxybenzotriazole (HOBt)/*N*,*N*-diisopropylethylamine (DIEA) (HBTU/HOBt/DIEA) was used as the coupling system to form the amide bonds. The use of 4 equiv of aa during 1 h guaranteed quantitative coupling in all cases. Fmoc group removal was accomplished by treatment with piperidine–*N*,*N*-dimethylformamide (DMF) (1:4) (2 × 1 min; 2 × 5 min). After incorporation of the third residue, Fmoc quantification proved the absence of DKP formation.

Once the linear precursor was fully assembled, it was cleaved from the resin (dichloromethane (DCM)–trifluoroacetic acid (TFA) (98:2), 5 × 2 min) and collected in water to prevent the loss of the side chain protecting groups (*tert*-butyloxycarbonyl (Boc) and ^{t}Bu). After lyophilization of the precursor, the macrolactamization reaction was undertaken for 3 h by means of benzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP)/1-hydroxy-7-azabenzotriazole (HOAt)/DIEA (2:1:4) in DCM–DMF (95:5) at pH 8 and at diluted conditions (10^{-4} M) to prevent oligomerization. Finally, all the side chain protecting groups were removed by treatment with TFA–H_2O (95:5) for 1 h, and the crude product was purified by reversed-phase semipreparative HPLC to obtain phakellistatin 19 in 22% overall yield (see Supporting Information).

Chemical and Spectral Validation. To verify the chemical identity of the product obtained, samples of synthetic and natural phakellistatin 19 were dissolved in H_2O–acetonitrile (ACN) (1:1) and analyzed by reversed-phase high performance liquid chromatography photodiode array (HPLC-PDA) using a C18 analytical column (Figure 2a,b). The two samples were then coeluted using a flat long gradient (Figure 2c). A single peak was obtained, indicating the chemical equivalence of the two samples.

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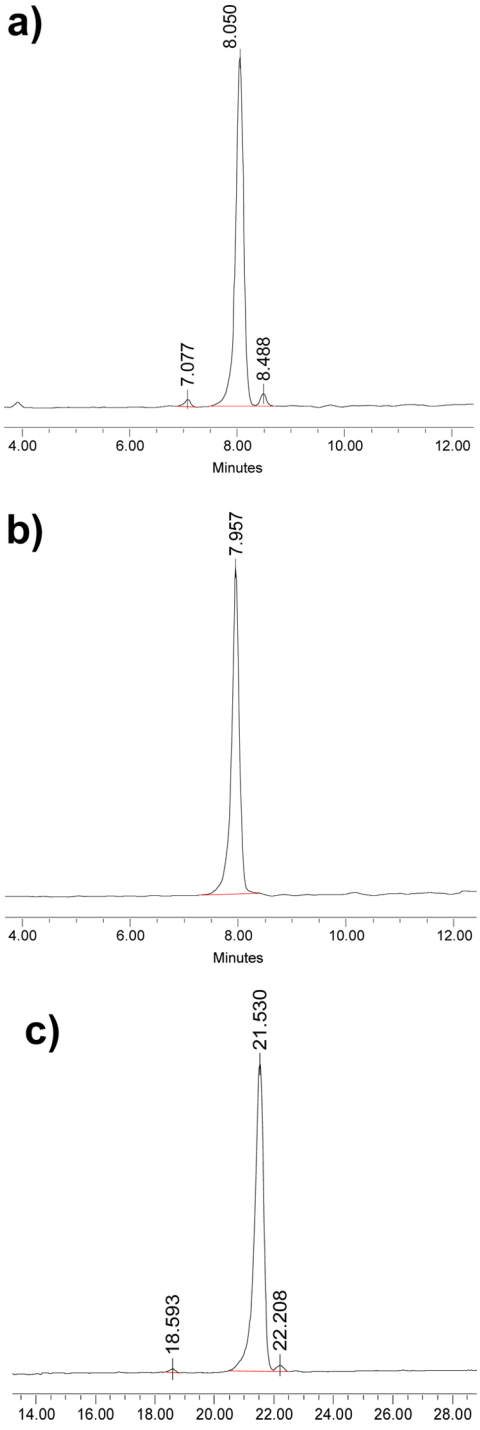

Figure 2. HPLC-PDA analysis of (a) synthetic phakellistatin 19, (b) natural phakellistatin 19, and (c) both natural and synthetic phakellistatin 19, coelution. Conditions were the following: Symmetry C18 reversed-phase analytical column (5 μm × 4.6 mm × 150 mm); linear gradient from 45% to 75% of ACN over 15 min for (a) and (b) and from 35% to 55% of ACN over 30 min for the coelution experiment.

HRMS data for the natural and synthetic phakellistatin 19 also matched perfectly. In addition, comparison of monodimensional ^{1}H and homonuclear bidimensional spectra of natural and synthetic phakellistatin 19 in CD_3OD at 298 K proved their spectral equivalence (Table 1). Only minor differences were detected when comparing complete ^{1}H assignment of the two peptides. Rotating frame Overhauser effect spectroscopy

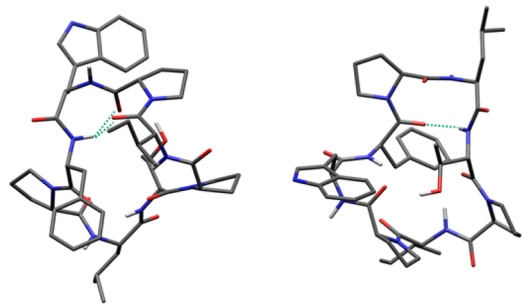

Figure 3. Energetically minimized structure of phakellistatin 19 obtained after application of a restricted SA protocol. The two checked hydrogen bonds are highlighted in dashed green lines.

(ROESY) cross-peaks between H_α-AA^{i+1} and H_δ-Pro^i were found for all the Pro moieties in both natural and synthetic phakellistatin 19, confirming the *trans* geometry of the prolyl peptide bonds in all cases. Moreover, ROE cross-peaks involving the side chains of Phe and Ile proved the presence of key hydrophobic interactions between these two residues in both peptides. Finally, the existence of minor conformers is detected for synthetic and natural phakellistatin 19. The occurrence of these conformers in such a small percentage prevents their assignment.

However, as expected, biological evaluation of synthetic phakellistatin 19 against three human cancer cell lines did not provide the same cytotoxicity as for its natural counterpart (see Table 2).

Structural Elucidation. Exhaustive NMR studies further proved that the three Pro residues in synthetic phakellistatin 19 adopted the *trans* geometry in all solvents (dimethyl sulfoxide (DMSO)-d_6, $CDCl_3$, CD_3OD). The structure of phakellistatin 19 was calculated by applying a restricted simulated annealing (SA) protocol. ROESY cross-peaks volumes (in DMSO-d_6) were suitably corrected and converted into interatomic distances which were used as experimental restrictions in the SA. Furthermore, variable-temperature nuclear magnetic resonance (VTNMR) analysis strongly pointed out the likely participation of the Phe and Thr amide protons in two intramolecular hydrogen bonds. They were also taken into account when undertaking the SA. The minimized structure of synthetic phakellistatin 19 (Figure 3) presents a β-turn stabilized by the hydrogen bond ThrN**H**−**O**CPhe (the measured distance is 2.06 Å) and with the residues Pro[1] and Leu placed at the positions i + 1 and i + 2 respectively.[12] A second hydrogen bond is observed, PheN**H**−**O**CPro[4] (1.95 Å), that would stabilize a γ-turn involving the residues Phe, Trp, and Pro[4] in the positions i, i + 1, and i + 2, respectively. The ϕ_{i+1} and ψ_{i+1} angles measure 78° and −56°, respectively, on the minimized structure. These values perfectly match the ones established for a γ-turn: 70 to 95 for ϕ_{i+1} and −75 to −45 for ψ_{i+1}. The region around the γ-turn resembles a hairpin motif. It comprises two antiparallel strains linked by means of hydrogen bonds and orienting the hydrophobic side chains of the Phe and Ile residues into the same direction. Van der Waals interactions between these two side chains help to stabilize the structural motif. Moreover, Trp's side chain points to the upper region of the γ-turn and is completely exposed to the solvent, suggesting its possible participation in the pharmacophore. The whole structure is highly folded and, somehow, draws a characteristic chair shape. Remarkably, the key ROESY cross-peaks between H_β-Ile and H_β-Phe and between H_γ-Ile and H_β-Phe are found in both synthetic and natural phakellistatin 19.

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Table 1. ^{1}H NMR Spectral Assignment of Natural and Synthetic Phakellistatin 19 in CD_3OD

	^{1}H (ppm) natural peptide	^{1}H (ppm) synthetic peptide		^{1}H (ppm) natural peptide	^{1}H (ppm) synthetic peptide		^{1}H (ppm) natural peptide	^{1}H (ppm) synthetic peptide
Pro^1			H_2	6.72 (d, 1.7)	6.73 (s)	Pro^6		
H_α	4.22	4.22	H_4	7.48 (d, 7.9)	7.49 (d, 7.9)	H_α	4.56 (dd, 8.8, 5.5)	4.57 (dd, 8.7, 5.4)
H_β	2.30, 1.89′	2.30, 1.90′	$H_{5/6}$	7.09 (t, 7.2)	7.09 (t, 7.1)	H_β	2.31, 1.96′	2.33, 1.97′
H_γ	2.07	2.10	$H_{6/5}$	7.00 (m)	7.00 (m)	H_γ	1.95	1.95
H_δ	3.96, 3.75′	3.97 (t, 8.1), 3.75′	H_7	7.35−7.24	7.35−7.24	H_δ	3.83, 3.67′	3.83, 3.68′
Phe^2 NH		8.19 (d, 9.8)	NH_{ind}	10.29		Thr^7 NH		7.93 (d, 9.6)
H_α	5.27	5.27 (td, 9.4, 5.0)	Pro^4			H_α	5.06	5.07 (dd, 9.7, 4.0)
H_β	3.01	3.02	H_α	3.98	3.98	H_β	4.36	4.36
$H_{2/6}$	7.27−7.24	7.28−7.24	H_β	2.04, 1.77′	2.04, 1.79′	H_γ	1.13 (d, 6.3)	1.14 (d, 6.3)
$H_{3/5}$	7.35−7.30	7.35−7.31	H_γ	1.94, 1.87′	1.96, 1.87′	Leu^8 NH		8.74 (d, 6.7)
H_4	7.35−7.24	7.35−7.24	H_δ	4.05, 3.41′	4.05, 3.41′	H_α	3.76	3.76
Trp^3 NH			Ile^5 NH		8.56 (d, 8.7)	H_β	2.36, 1.77′	2.37, 1.78′
H_α	4.24	4.24	H_α	4.25	4.26	H_γ	1.58	1.58
H_β	3.40, 3.16′	3.42 (dt, 14.9, 7.7), 3.17′ (14.9, 4.8)	H_β	2.06	2.06	H_δ	0.96 (d, 6.7)	0.97 (d, 6.7)
			H_γ	1.47, 1.13′	1.47, 1.13′		0.93 (d, 6.5)	0.93 (d, 6.5)
				0.60 (d, 6.8)	0.61 (d, 6.8)			
			H_δ	0.80 (t, 7.4)	0.81 (t, 7.4)			

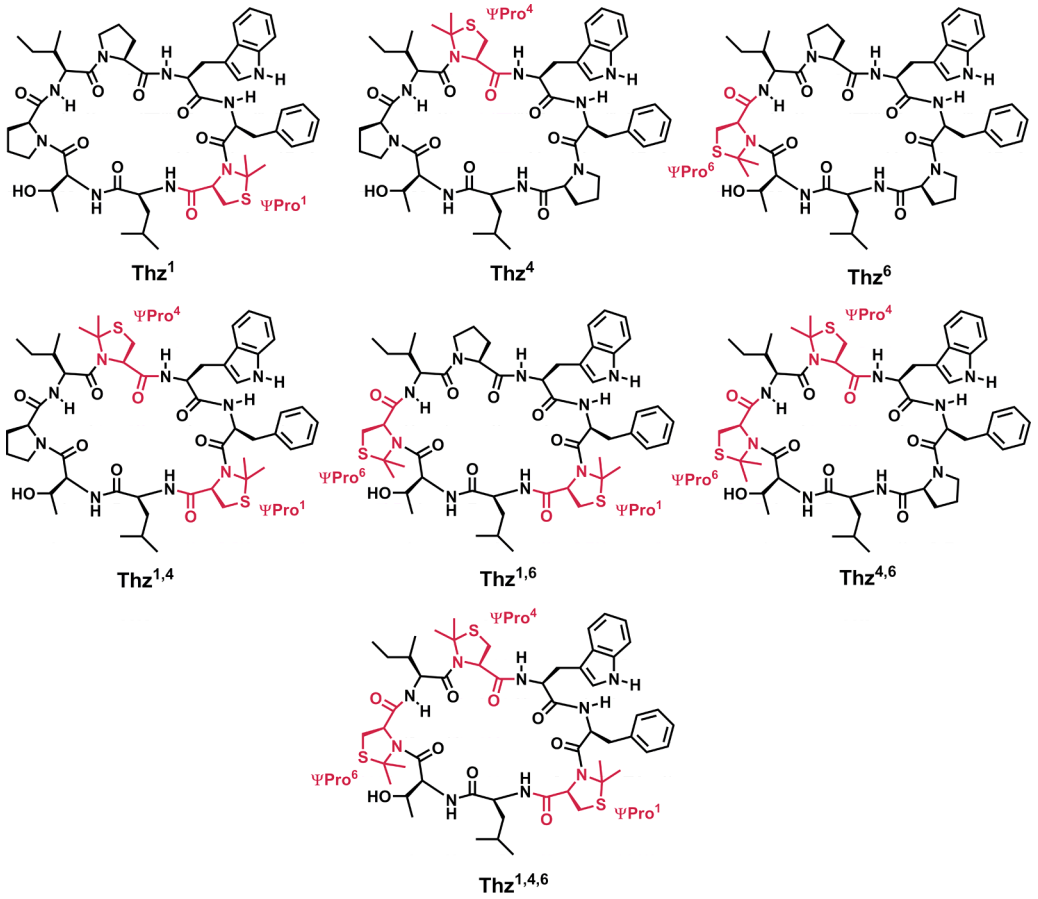

Figure 4. Chemical structures of Cys($\Psi^{Me,Me}$pro) analogues of phakellistatin 19. The residues Cys($\Psi^{Me,Me}$pro) are highlighted in dark red.

Influence of the pH on the Geometries of Prolyl Peptide Bonds. To discard a possible influence of the pH in the biological behavior (in vitro assays are carried out in aqueous media), a spectrally comparable and H_2O-soluble analogue was designed on the basis of the previous described minimized structure, with a positively charged Orn at the Leu's position. Its synthesis was successfully achieved following the synthetic strategy developed for phakellistatin 19, and the biological assays revealed no significant cytotoxic properties. Once the Orn analogue was proven to be H_2O-soluble, its spectral equivalence with synthetic phakellistatin 19 was checked by comparison of ^{1}H NMR spectral assignments of the two peptides in DMSO-d_6, confirming small differences. After complete NMR characterization at pH 5.95 and 8.12, *trans* isomerism was confirmed for

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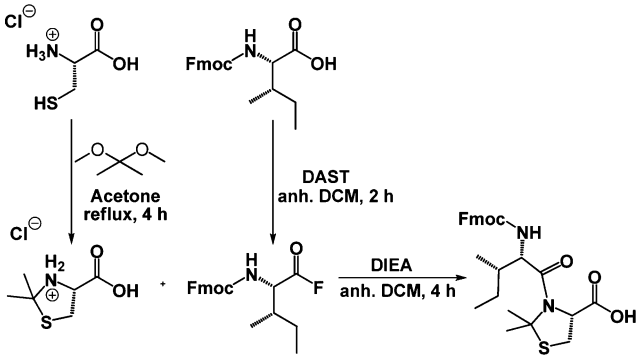
Scheme 2. Synthetic Strategy To Obtain Cys($\Psi^{Me,Me}$pro)-Containing Dipeptides[a]



[a]Fmoc-Ile-Cys($\Psi^{Me,Me}$pro)-OH synthesis is represented.

Table 2. In Vitro Results for Natural Phakellistatin 19, Synthetic Phakellistatin 19, and Thz Analogues[a]

		NSCLC (Lung) A549	Colon HT-29	Breast MDA-MB-231
Natural phakellistatin 19	GI_{50}	4.41E-7	4.62E-7	5.15E-7
	TGI	1.16E-6	6.72E-7	1.47E-6
	LC_{50}	>1.05E-5	>1.05E-5	>1.05E-5
Synthetic phakellistatin 19	GI_{50}	n. d.	n. d.	n. d.
	TGI	n. d.	n. d.	n. d.
	LC_{50}	n. d.	n. d.	n. d.
Thz^{1}	GI_{50}	>1.00E-5	5.71E-6	6.11E-6
	TGI	>1.00E-5	>1.00E-5	>1.00E-5
	LC_{50}	>1.00E-5	>1.00E-5	>1.00E-5
Thz^{4}	GI_{50}	>1.00E-5	>1.00E-5	>1.00E-5
	TGI	>1.00E-5	>1.00E-5	>1.00E-5
	LC_{50}	>1.00E-5	>1.00E-5	>1.00E-5
Thz^{6}	GI_{50}	>1.00E-5	4.01E-6	2.70E-6
	TGI	>1.00E-5	4.21E-6	4.11E-6
	LC_{50}	>1.00E-5	4.61E-6	6.01E-6
$Thz^{1,4}$	GI_{50}	>9.58E-6	3.16E-6	5.65E-6
	TGI	>9.58E-6	6.61E-6	>9.58E-6
	LC_{50}	>9.58E-6	>9.58E-6	>9.58E-6
$Thz^{1,6}$	GI_{50}	3.45E-6	1.82E-6	1.72E-6
	TGI	7.85E-6	2.20E-6	2.01E-6
	LC_{50}	>9.58E-6	2.68E-6	2.30E-6
$Thz^{4,6}$	GI_{50}	3.64E-6	1.82E-6	2.20E-6
	TGI	>9.58E-6	2.11E-6	3.35E-6
	LC_{50}	>9.58E-6	2.39E-6	5.08E-6
$Thz^{1,4,6}$	GI_{50}	1.47E-6	1.38E-6	1.65E-6
	TGI	1.65E-6	1.74E-6	1.93E-6
	LC_{50}	1.83E-6	2.11E-6	2.20E-6

[a]n.d. means not detected. GI_{50}, compound concentration that produces 50% of cell growth inhibition compared to control cultures. TGI, total cell growth inhibition. LC_{50}, compound concentration that produces 50% of net cell killing. IC_{50}, compound concentration that produces 50% inhibition of biological activity of cancer cells. Antimitotic activity for natural phakellistatin 19 has also been described: IC_{50} = 4.20 × 10^{-7} to 8.40 × 10^{-8} M (see Supporting Information).

the three prolines also in these conditions, proving that no conformational change at the Pro linkages occurred in acidic or basic media.

In view of all these results and considering the presence of minor peaks in the NMR spectra and the HPLC-PDA chromatogram of natural phakellistatin 19 (see Figure 2a and Supporting Information), it is plausible that a cytotoxic agent, present in very small amounts, would be mainly responsible for the high biological activity.

Phakellistatin 19 as a Hit for a Medicinal Chemistry Program. Despite the low biological activity of synthetic phakellistatin 19, we believe that its structure is enough interesting as a starting point for the development of a medicinal chemistry program. As a first step, we decided to cover a new conformational scenario in the search of an increased biological activity. To this end, chemical modification was undertaken to access analogues of phakellistatin 19 with induced *cis*-isomerism by replacing Pro by Cys($\Psi^{Me,Me}$pro). In 1992, Mutter et al. described new Pro surrogates easily accessed by means of cyclocondensation of the amino acids Ser, Thr, or Cys with aldehydes or ketones.[13] These pseudo-prolines (ΨPro or Xaa($\Psi^{R,R'}$pro)) acted as structure-disrupting agents, preventing peptide aggregation and self-association and therefore increasing the efficiency of peptide synthesis.[14] Furthermore, they can be used as removable turn inducers, facilitating the cyclization of linear peptides.[15] Finally, their introduction into a peptide sequence contributes to the modulation of the peptide's biological and pharmacokinetic properties.[16]

Interestingly, the *cis* to *trans* ratio at the Xaa^{i+1}-Xaa($\Psi^{R,R'}$pro)^{i} amide bond, as well as the lability of pseudo-prolines to acid, can be modulated. Thus, Cys-derived pseudo-prolines exhibit a larger Pro effect enhancement in comparison to the ΨPro obtained from Thr and Ser.[16] Moreover, both the stereochemistry and the degree of substitution at the 2-C ΨPro position are crucial factors determining the *cis* content along the imidic bond.[17,18]

Detailed NMR studies confirmed that 2,2-dimethylated derivatives show a higher percentage of *cis* geometry at the Xaa-ΨPro peptide bond.[17,18] Thus, it was finally decided that Cys($\Psi^{Me,Me}$pro) would replace Pro to strongly enhance the *cis* conformer at the Xaa^{i+1}-Cys($\Psi^{Me,Me}$pro)^{i} peptide bonds in synthetic phakellistatin 19. With this purpose in mind, a small library of seven peptides with the Pro moieties replaced by Cys($\Psi^{Me,Me}$pro), covering all the possibilities, was designed and biologically tested (Figure 4).

The synthetic strategy previously validated for phakellistatin 19 could not be directly applied to obtain the pseudo-proline-containing analogues, since the extremely hindered Cys-($\Psi^{Me,Me}$pro) was not acylated under any conditions (1-[(1-cyano-2-ethoxy-2-oxoethylideneaminooxy)dimethylamino-morpholinomethylene)]methanaminium hexafluorophosphate (COMU)/OxymaPure/DIEA, 1-[bis(dimethylamino)-methylene]-1*H*-1,2,3-triazolo-[4,5-*b*]pyridinium hexafluoro-phosphate 3-oxide (HATU)/HOAt/DIEA, PyBOP/HOAt/DIEA, *N*,*N*,*N*′,*N*′-tetramethylchloroformamidinium hexafluor-ophosphate (TCFH)/HOAt/DIEA, or Fmoc-aa-F) applying microwave and other reagents and techniques. Taking this into consideration, all approaches including solid-phase acylation of Cys($\Psi^{Me,Me}$pro) were discarded, and the synthesis in solution of dipeptides containing the Cys($\Psi^{Me,Me}$pro) at the *C*-terminus was faced.[19] Although coupling of Fmoc-aa-F on Cys($\Psi^{Me,Me}$pro) was not achieved on solid phase, it did work in solution to form the corresponding dipeptides (Scheme 2).

With all dipeptides in hand, a modified synthetic scheme on solid phase was achieved to access all the Cys($\Psi^{Me,Me}$pro)-containing analogues. For the synthesis of Thz^{1}, $Thz^{1,4}$, $Thz^{1,6}$, and $Thz^{1,4,6}$ analogues, the starting/cyclization point was changed to the Thr-Leu linkage to prevent direct incorporation of the dipeptide onto the resin. The synthesis of all the

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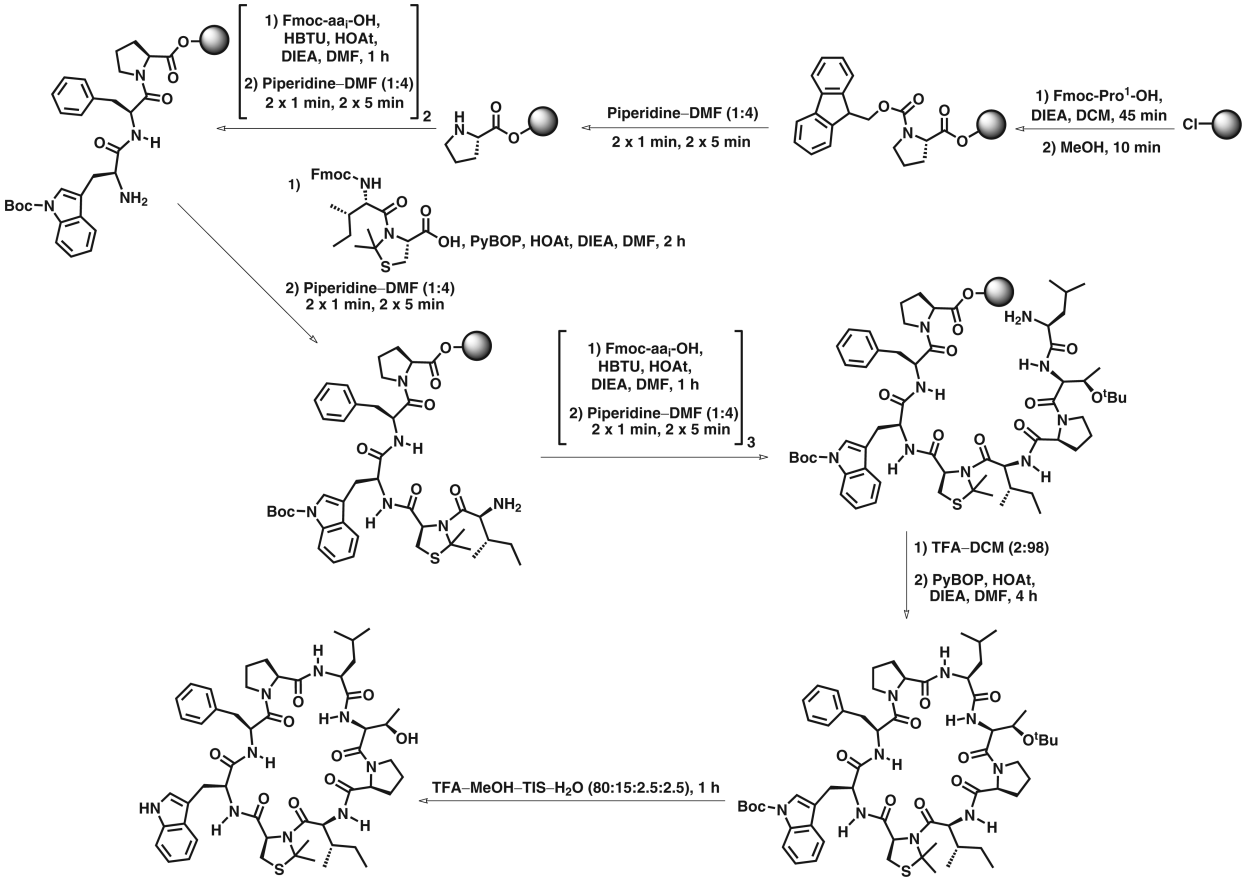
Scheme 3. Synthetic Strategy for the Thz[4] Phakellistatin 19 Analogue as Example for the Rest of Cys($\Psi^{Me,Me}$pro)-Containing Peptides



Cys($\Psi^{Me,Me}$pro)-containing analogues was successfully achieved. A representative scheme for Thz[4] analogue is shown (Scheme 3).

Data obtained from the biological assays showed that replacement of Pro[6] by a Cys($\Psi^{Me,Me}$pro) caused a significant increase in cytotoxicity (Table 2). Moreover, a more rigid structure produced by the presence of an increasing number of Cys($\Psi^{Me,Me}$pro) residues also increased the bioactivity of the compound. For a better understanding of these results, we performed a structural study of the library by means of NMR. CD_3OH was chosen as the working solvent because it favors the coexistence of conformers less than $CDCl_3$ does.

^{1}H NMR spectra of the most active monosubstituted analogue Thz[6] in CD_3OH were recorded at two temperatures: 278 and 308 K. Analysis of the indolic region (between 10 and 11 ppm) of the spectra provided an idea of the complex conformational equilibrium in which this analogue was involved. Replacement of one single Pro residue by a Cys($\Psi^{Me,Me}$pro) dramatically modified the *cis*–*trans* isomerism at the Pro linkages, thus altering the conformational panorama. In CD_3OD at 298 K, synthetic phakellistatin 19 appeared as a single major conformer with all Pro in *trans*, while Thz[6] presented in CD_3OH at least seven conformers (five major conformers and two minor conformers) at 278 K and at least four conformers (two of them in a fast equilibrium) at 308 K. Any attempt to assign ^{1}H appeared extremely challenging. Thus, two presumably less flexible analogues, Thz[1,4] and Thz[1,6], were examined.

As detected by NMR, Thz[1,4] in CD_3OH at 298 K presented two major conformers, in a conformer 1 to conformer 2 ratio of 58:42. Exhaustive analysis of ^{1}H, correlation spectroscopy (gCOSY), total correlation spectroscopy (TOCSY), nuclear Overhauser effect spectroscopy (NOESY), ROESY, and heteronuclear single-quantum correlation spectroscopy (gHSQC) experiments allowed ^{1}H NMR spectral assignment of both conformers.

The gHSQC experiment enabled us to assign the key carbon atoms of the Pro moiety C_β and C_γ. For Pro[6] of conformer 1, $\delta_{C\beta}$ = 33.095 ppm and $\delta_{C\gamma}$ = 22.643 ppm, meaning that $\Delta\delta_{C\beta\text{-}C\gamma}$ = 10.452 ppm. For Pro[6] of conformer 2, $\delta_{C\beta}$ = 29.943 ppm and $\delta_{C\gamma}$ = 26.791 ppm, meaning that $\Delta\delta_{C\beta\text{-}C\gamma}$ = 3.152 ppm. According to these data, Pro[6] adopted *cis* isomerism in conformer 1 and *trans* isomerism in conformer 2. A large NOE cross-peak between H_α-Thr and H_δ-Pro[6] in conformer 2 and ROE cross-peak between H_α-Thr and H_α-Pro[6] in conformer 1 also supported the assigned isomerism at the Thr-Pro[6] linkage for the two conformers.

As expected, both Cys($\Psi^{Me,Me}$pro)[1] and Cys($\Psi^{Me,Me}$pro)[4] adopted *cis* isomerism in all conformers. Large NOE cross-peaks between H_α-Phe and H_α-Cys($\Psi^{Me,Me}$pro)[1] and between H_α-Ile and H_α-Cys($\Psi^{Me,Me}$pro)[4] served as confirmation.

The Thz[1,6] analogue showed more confusing spectroscopic data. Again, two major distinct conformers were detected. ^{1}H and ^{13}C NMR spectral assignment of the 16 residues was accomplished, but the cross-peaks of the ROESY spectra did not provide enough information to perform complete sequential assignment of the two conformers.[20] However, the two Pro moieties were fully assigned and their geometry was identified (see Supporting Information).

For conformer 1, it was found that $\Delta\delta_{C\beta\text{-}C\gamma}$ = 9.54 ppm, suggesting a *cis* geometry of the amide bond Ile-Pro[4]. On the

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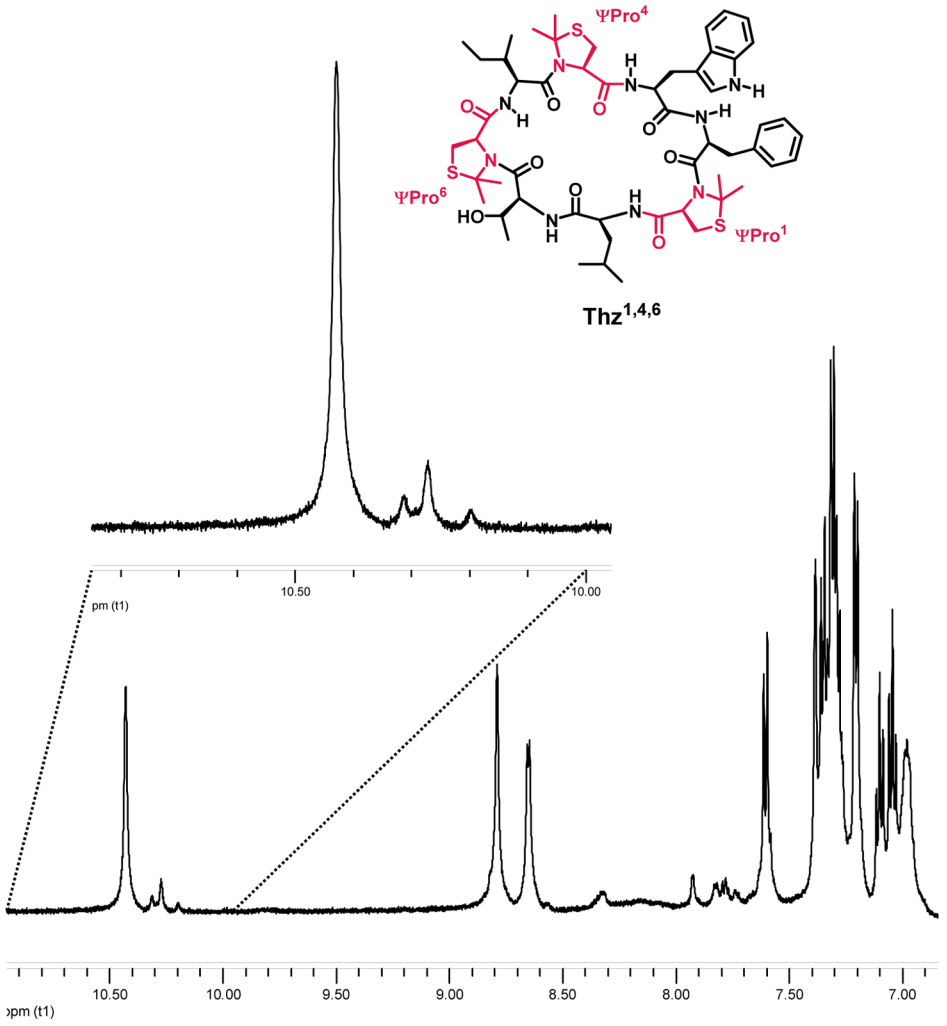

Figure 5. ^{1}H NMR spectrum of Thz^{1,4,6} analogue in CD_3OH at 298 K. The region between 10 and 11 ppm is enlarged.

contrary, for conformer 2, $\Delta\delta_{C\beta\text{-}C\gamma}$ = 2.03 ppm strongly pointed to a *trans* prolyl peptide bond. The ROESY cross-peaks detected between H_α-Ile′ and $H_\delta/H_{\delta'}$-Pro$^{4'}$ for conformer 2 also supported the *trans* isomerism. For conformer 1, no ROE cross-peak between H_α-Ile and H_α-Pro^4 was detected, probably because of the low quality of the ROESY spectra. The *cis* geometry of the Xaa-Cys($\Psi^{Me,Me}$pro) linkages was confirmed by the cross-peaks between H_α-Thr and H_α-Cys($\Psi^{Me,Me}$pro) and between H_α-Phe and H_α-Cys($\Psi^{Me,Me}$pro). All the NMR experiments were recorded in CD_3OH at 273 K.

Finally, the Thz^{1,4,6} analogue, with the three Pro residues replaced by Cys($\Psi^{Me,Me}$pro), was the most conformationally restricted peptide, as confirmed by the presence of one major (89%) and three minor conformers (Figure 5). NMR studies showed that all Cys($\Psi^{Me,Me}$pro) adopted *cis* isomerism, as proved by the NOE cross-peaks detected between the protons H_α-Phe and H_α-Cys($\Psi^{Me,Me}$pro)^1, H_α-Ile and H_α-Cys($\Psi^{Me,Me}$pro)^4, H_α-Thr and H_α-Cys($\Psi^{Me,Me}$pro)^6, H_β-Phe and H_α-Cys($\Psi^{Me,Me}$pro)^1, H_β-Ile and H_α-Cys($\Psi^{Me,Me}$pro)^4, and H_β-Thr and H_α-Cys-($\Psi^{Me,Me}$pro)^6.

Together, the biological and structural data suggest that Pro^6 plays a crucial role in the structure of phakellistatin 19 analogues and has a direct effect on the bioactivity exerted by the ΨPro-containing peptides. Pro replacement by Cys($\Psi^{Me,Me}$pro) causes a significant gain of steric hindrance due to the presence of the two extra Me groups, and also an alteration of the hydrogen donors and acceptors pattern, as a sulfur atom (a hydrogen acceptor) is introduced. However, the bioactivity results showed a noticeable trend from the monosubstituted analogue Thz^6 (lower activity) toward the trisubstituted analogue Thz^{1,4,6} (highest activity). Moreover, only small differences are observed between Thz^1 and Thz^6. Thus, we propose that this difference is caused by a structural issue (gain of *cis* geometry) rather than by the aa^6 being the pharmacophore of phakellistatin 19 analogues.

Moreover, as confirmed by NMR analysis, an increasing number of Cys($\Psi^{Me,Me}$pro) in the phakellistatin 19 structure entails a gain of structural rigidity. The Thz^{1,4,6} analogue not only was the most active one but also was the most rigid, with a major conformer accounting for 89% of the mixture with all the Xaa^{i+1}-Cys($\Psi^{Me,Me}$pro)^i linkages adopting the *cis* isomerism. A significantly more active all-*cis* analogue of phakellistatin 19 strongly suggests that the *cis*−*trans* isomerism at the Pro linkages makes a crucial contribution to the bioactivity displayed by the ΨPro-containing analogues of phakellistatin 19.

CONCLUSIONS

The introduction of $\Psi^{Me,Me}$pro residues in a cyclic peptide such as phakellistatin 19 increases the percentage of *cis* conformation in the final peptides, and this translates into enhanced biological activity. A correlation between the number of $\Psi^{Me,Me}$pro units

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introduced and the enhanced cytotoxic activity was also observed, the peptide containing the three $\Psi^{Me,Me}$pro residues showing the highest activity. In this regard, we envisage that the use of $\Psi^{Me,Me}$pro moieties will be widely adopted to increase the biological activity of cyclic peptides. Furthermore, other families of Pro-rich cyclic peptides should be revised under this new perspective.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic protocols and characterization data of phakellistatin 19, epimers, and analogues. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

ACN, acetonitrile; Alloc, allyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; 2-CTC, 2-chlorotrityl chloride (Barlos) resin; COMU, 1-[(1-(cyano-2-ethoxy-2-oxoethylideneaminooxy)-dimethylaminomorpholinomethylene)]methanaminium hexafluorophosphate; COSY, correlation spectroscopy; DCM, dichloromethane; DIEA, *N*,*N*-diisopropylethylamine; DIPCDI, *N*,*N*′-diisopropylcarbodiimide; DKP, diketopiperazine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; ESMS, electrospray mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; HATU, 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium hexafluorophosphate 3-oxide; HBTU, 1-[bis(dimethylamino)methylene]-1*H*-benzotriazolium hexafluorophosphate 3-oxide; HOAt, 1-hydroxy-7-azabenzotriazole; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; HRMS, high-resolution mass spectrometry; HSQC, heteronuclear single-quantum correlation spectroscopy; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy; PDA, photodiode array; PyBOP, benzotriazol-1-yloxytris(pyrrolidino)-phosphonium hexafluorophosphate; ROESY, rotating frame Overhauser effect spectroscopy; ΨPro, pseudo-proline; TBME, *tert*-butyl methyl ether; ^{t}Bu, *tert*-butyl; TCFH, *N*,*N*,*N*′,*N*′-tetramethylchloroformamidinium hexafluorophosphate; TFA, trifluoroacetic acid; TIS, triisopropylsilane; TOCSY, total correlation spectroscopy; VTNMR, variable-temperature nuclear magnetic resonance

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